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Published in:
Applied Microbiology and Biotechnology

DOI:
10.1007/s00253-011-3312-5

Publication date:
2011

Appl Microbiol Biotechnol (2011) 90:2027–2036
DOI 10.1007/s00253-011-3312-5

BIOENERGY AND BIOFUELS

Enhancing Jatropha oil extraction yield from the kernels assisted by a xylan-degrading bacterium to preserve protein structure

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Received: 10 February 2011 / Revised: 5 April 2011 / Accepted: 5 April 2011 / Published online: 10 May 2011

Abstract We investigated the use of bacterial cells isolated from paddy crab for the extraction of oil from Jatropha seed kernels in aqueous media while simultaneously preserving the protein structures of this protein-rich endosperm. A bacterial strain—which was marked as MB4 and identified by means of 16S rDNA sequencing and physiological characterization as either *Bacillus pumilus* or *Bacillus altitudinis*—enhanced the extraction yield of Jatropha oil. The incubation of an MB4 starter culture with preheated kernel slurry in aqueous media with the initial pH of 5.5 at 37 °C for 6 h liberated 73% *w*/*w* of the Jatropha oil. Since MB4 produces xylanases, it is suggested that strain MB4 facilitates oil liberation via degradation of hemicelluloses which form the oil-containing cell wall structure of the kernel. After MB4 assisted oil extraction, SDS-PAGE analysis showed that the majority of Jatropha proteins were preserved in the solid phase of the extraction residues. The advantages offered by this process are: protein in the residue can be further processed for other applications, no purified enzyme preparation is needed, and the resulting oil can be used for biodiesel production.

Keywords *Jatropha curcas* · Aqueous oil extraction · *Bacillus pumilus* · *Bacillus altitudinis* · Protease · Xylanase

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Introduction

Jatropha curcas is a well-known plant for the high fat and protein content of its seed ranging between 45% and 55% *w*/*w* and 20–30% *w*/*w* of the kernels, respectively (Gubitz et al. 1999; Lestari et al. 2010). This oil is economically attractive due to its potential application in biodiesel (Lin et al. 2003; Martinez-Herrera et al. 2006). In addition, the kernel contains 28% protein, which has been extensively studied for food and non-food application (Gubitz et al. 1999; Lestari et al. 2010; Lin et al. 2003; Martinez-Herrera et al. 2006). Lestari extracted more than 80% of the protein from the kernels and addressed some potential applications of the isolated protein in various fields such as adhesives, coatings, and chemicals (Lestari et al. 2010). Therefore, with respect to the overall economy of Jatropha cultivation, it is interesting to find a commercial use for both oil and protein. Protein recovery from the kernel requires aqueous extraction; hence, it is interesting to see if aqueous extraction can also be used for the release of oil.

The common method of oil production from oilseeds as feedstock for biodiesel involves pressing of seeds and oil purification (degumming, deacidification, dewaxing, dephosphorization, dehydration, etc.). These processes, together with esterification/transesterification, contribute to over 70% of the total biodiesel production costs (Shuit et al. 2010; Zeng et al. 2009).

Aqueous oil extraction (AOE) uses water as medium to facilitate oil liberation from oilseeds. AOE eliminates organic solvent consumption and so improves process economy (Barrios et al. 1990; Rosenthal et al. 1996). AOE also enables several purification steps such as degumming, deacidification, dewaxing, and dephosphorization to be carried out simultaneously within the extraction step (Caragay 1983) resulting in a more efficient process.

We demonstrated earlier that thermophilic strains isolated from the gut of paddy crabs, one of which was identified as *Bacillus licheniformis*, enhanced oil liberation up to 60% from aqueous *J. curcas* kernel, most likely via protein degradation (Marasabessy et al. 2010), which would be disadvantageous for protein recovery. In the present report, we confirmed that these thermophilic bacteria degraded extracted *J. curcas* protein. We also examined if preheating the kernels degraded the proteins in comparison to non-heated kernels by using SDS-PAGE analysis. Next, isolation and selection of mesophilic bacteria from the gut of paddy crabs were performed based on their ability to liberate oil from *J. curcas* preheated kernel slurry. The aim was to obtain other microorganisms able to liberate oil without affecting the protein structures. The best strain was used for aqueous oil extraction from *J. curcas* kernel. The molecular weight distribution of protein in the residue (water phase and solid phase) after microbial treatment was also investigated to examine protein integrity. The quality of recovered oil was analyzed and compared with those of standard values of feedstock for biodiesel.

Materials and methods

Materials

Jatropha seeds were harvested from *J. curcas* planted in Serpong, Indonesia (geocoordinates 6°21′31″ S, 106°40′33″ E). Kernels were obtained after removal of the shells. The sun-dried kernels were stored at 4 °C until used. Paddy crabs were collected from bunds of a paddy field located in Pamulang, Indonesia (geocoordinates 6°20′52″ S, 106°42′20″ E). All chemical reagents, unless otherwise specified, were of analytical grade.

J. curcas kernels slurry preparation

The kernels (500 g) were autoclaved at 121 °C for 15 min and then dried at 60 °C overnight. The kernels were milled and sieved through a strainer with 1.0 mm pore diameter. To prepare the preheated kernel slurry, 25 g milled kernel was homogenized with 125 g purified water (milli-Q) for 5 min using a Waring Blender. The weight ratio of solid material to water in the slurry was 1:5. Under constant stirring—to keep the slurry homogenous—12 g of kernel slurry (equivalent to 2 g kernel) was used for the extraction of oil.

Protein extractions from kernels

Protein extraction was carried out by extracting 1 g of sample with 30 ml solvent for 30 min in 50 ml capped centrifuge tubes. The mixing was conducted at room temperature by using a rotary mixer. The extracting solvents were water, NaCl 1.0 M, and NaOH 0.055 M as described previously (Lestari et al. 2010). Solid–liquid separation was conducted at 4,000×*g* for 15 min by using a SORVALL6+ centrifuge.

Evaluation of protein degradation by paddy crab bacteria

A mixture of 15 g/L Agar–agar (Merck) and 10 g/L of *J. curcas* seed protein having a purity of ca. 83% *w*/*w* or 10.0 g/L of casein (Merck) in water was boiled to dissolve agar. After autoclaving (121 °C, 15 min), 15 ml protein–agar solution was aseptically poured in a sterile petri dish and brought to solidify overnight. The wells in protein–agar media were made by using a sterile rubber cork having a diameter of 9 mm. Two milliliters of a 24-h old bacterial starter culture was centrifuged at 20,000 rpm for 5 min. The supernatant was filtered through a 0.22-μm bacterial filter (Millipore), after which 50 μl of the filtrate (bacterial crude extract) was pipetted into the well. The plates were placed at 4 °C overnight to let the extract absorb into the protein–agar media, followed by incubation at 37 °C for 6 h and at 45 °C for 6 h. Clear zones surrounding the well indicating protein solubilization (degradation) by bacterial proteases were observed. A thermostable bacterial neutral protease from *Bacillus thermoproteolyticus* (Protex 14 L, Genencor) at 200x dilution was used as the positive control, while the preheated bacterial crude extracts and preheated Protex 14 L (100 °C, 10 min), respectively, were used as negative controls.

Jatropha oil extraction by paddy crab paste

The crab paste was prepared as described previously (Marasabessy et al. 2010). To extract oil, 2.0 g of crab paste was mixed with 30.0 g of kernel slurry and incubated

in a orbital shaker at 37 °C, 150 rpm for 24 h. Antibiotics were applied in some samples as described previously and the extracted oil was assayed gravimetrically (Marasabessy et al. 2010).

Isolation of mesophilic bacteria from paddy crabs

For the isolation of bacteria, one loop of crab paste was streaked out aseptically on a nutrient agar (NA) medium plate (Merck) and incubated at 37 °C for 24 h. Well separated colonies were picked up, subcultured, and maintained on NA slants (37 °C for 24 h).

Selection of mesophilic bacteria for Jatropha oil extraction

Under constant stirring, 12 g of preheated kernel slurry (equivalent to 2 g kernel) was weighed out in a 100-ml flask. This was inoculated with 2 ml bacterial suspension, prepared by suspending cells of a bacterial culture grown on an NA agar slant (in a tube having 1.5 cm diameter and 12 cm length) with 2 ml sterile water. The mixture was incubated at 37 °C and 150 rpm for 24 h using an Innova 44 Incubator Shaker (New Brunswick), after which it was centrifuged at 7,400×*g* for 15 min. The extracted oil was assayed gravimetrically (Marasabessy et al. 2010). Control experiments were performed using exactly the same treatment, however without bacterial inoculation. A bacterial strain showing the best performance was identified by partial sequence of 16S rDNA as well as physiological tests conducted by DSMZ (Germany).

Microbial Jatropha oil extraction

Bacterial starter culture was prepared as described previously (Marasabessy et al. 2010), except that the nutrient broth medium (NB, Merck) was initially supplemented with 1.0% *w/v* milled Jatropha kernel before autoclaving. To extract the oil, 12.0 g of Jatropha kernel slurry was inoculated with 1.0 ml of the bacterial starter culture. Antibiotics were applied in some samples as described previously (Marasabessy et al. 2010). The mixture was shaken at 150 rpm and 37 °C. After incubation, the slurry was centrifuged at 7,400×*g* for 15 min. The free oil on the surface of the liquid in the centrifuge tube was assayed gravimetrically as reported previously (Marasabessy et al. 2010).

Detection of xylanase and glucanase activity in bacterial crude extracts

For xylanase detection, 15 μl bacterial crude extract was pipetted into a well (5 mm diameter) in an agar plate containing 0.2% Remazol Brilliant Blue Xylan (RBB-Xylan, Sigma) (Strauss et al. 2001). For cellulose detection, 50 μl bacterial crude extract was pipetted into a well (9 mm diameter) in an agar plate containing 0.4% carboxymethyl cellulose (CMC). The plates were placed at 4 °C overnight to let the extract absorb into the agar media, followed by incubation at 37 °C for 6 h (RBB-Xylan agar) and 48 h (CMC agar). The CMC agar plate was stained with 0.03% Congo Red, followed by destaining with 1 M HCl (Teather and Wood 1982). The clear zones surrounding the well indicate the hydrolysis of xylan and cellulose.

SDS-PAGE analysis

Molecular weight distribution of proteins was analyzed by using SDS-PAGE (NuPage Electrophoresis System with NuPage Novex Bis-Tris Gels 10% from Invitrogen).

Assay of total oil content, oil yield, and oil quality

The total oil content of the oilseeds was determined by Soxhlet method (AOAC 1984). The total oil content was 0.47 kg/kg Jatropha kernels. This amount was taken as 100% recovery of oil in the calculations of Jatropha oil yield in the extraction experiments. The free fatty acid and moisture content of the extracted oil was assayed by the titration method and the Karl Fischer method, respectively (AOAC 2002). The oxidative stability index (OSI) was assayed using 873 Biodiesel Rancimat apparatus from Metrohm.

Results

The effect of thermophilic crab bacteria on Jatropha protein integrity

In our previous publication, some thermophilic bacteria (namely BK21, BK22, and *B. licheniformis* strain BK23 isolated from paddy crabs) extracted up to 60% of the Jatropha oil from non-heated kernels after 24 h incubation at 50 °C under non-optimized conditions (Marasabessy et al. 2010). From the protein–agar plate experiment (Fig. 1), we found that those strains hydrolyzed both *J. curcas* kernel protein and casein upon incubation at 50 °C. BK21 showed the highest protease activity and *B. licheniformis* BK23 the lowest, as indicated by the size of the clearing zone diameter, for both types of protein. The bright clear zones proved that proteins available in the kernel were completely solubilized.

The effect of heat pretreatment on Jatropha protein integrity

Because we wanted to study the effect of crab's gut bacteria working at lower temperature on the extraction yield of oil

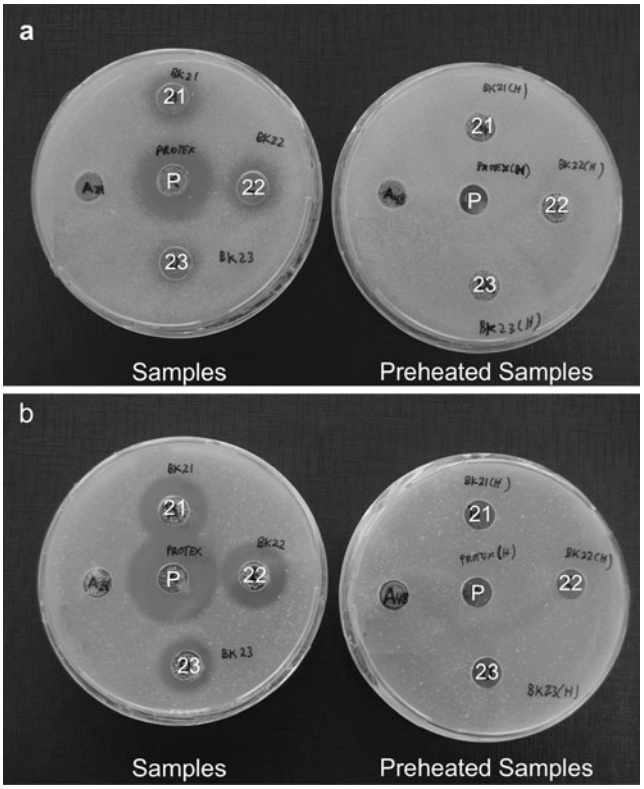

Fig. 1 Protein degradation ability of thermophilic bacterial crude extract of BK21, BK22, *B. licheniformis* strain BK23 on (**a**) Jatropha protein agar medium and (**b**) casein agar medium. These strains were isolated from paddy crabs (Marasabessy et al. 2010). A 200x dilution of Protex 14 L from Genencor (*P*) was the positive control. The preheated samples (100 °C, 10 min) were the negative controls (showing no clear zone)

from *J. curcas* kernels, internal factors within the kernels influencing oil liberation had to be minimized. Since the kernels contain microorganisms as well as seed enzymes which might interfere with the crab bacteria involved in oil liberation, we applied two different heat pretreatments on kernels, at 105 °C or 121 °C for 30 min, to deactivate enzymes and to kill microorganisms before being used for oil extraction. The proteins were extracted from the kernels and the extracts were subjected to SDS-PAGE analysis (Fig. 2). The solubility of proteins in water depends on various factors such as ionic strength and pH; therefore, 1 M NaCl and 0.055 M NaOH were also used as extractants besides water (Lestari et al. 2010). The protein pattern of the heat-treated kernels extracted with NaCl and NaOH was identical with that of the untreated kernels, showing that heat treatment at both 105 and 121 °C for 30 min did not affect the protein composition. Compared to the other samples, the untreated sample extracted with water is missing three small bands at molecular weights of approximately 20, 23, and 25 kDa, indicating that heat treatment increased the water solubility of the proteins.

Concluding, heat pretreatment did not have an effect on position and relative intensity of the different protein bands on the SDS-PAGE, indicating that no significant alteration of the chemical structures of the proteins occurred. We decided therefore to employ preheated kernels (by autoclaving at 121 °C for 15 min) for oil extraction in the subsequent experiments.

Effect of paddy crab paste on oil extraction

The presence of mesophilic bacteria in paddy crabs having a positive effect on Jatropha oil liberation was determined by incubating 2 g paddy crab paste with 30 g preheated kernel slurry (containing 5 g kernel) at 37 °C and 150 rpm for 24 h with and without addition of antibiotics (Fig. 3). Oil extraction in control experiments to which no crab paste was added resulted in 7% oil after 24 h. Addition of antibiotics to control samples also gave a low oil yield (4%). Incubation of preheated kernel slurry with crab paste and antibiotics significantly improved the oil yield to 62%. It is evident that paddy crab paste exhibits a strong effect towards oil liberation from Jatropha kernel. Furthermore, it was shown that excluding antibiotics from the crab–kernel

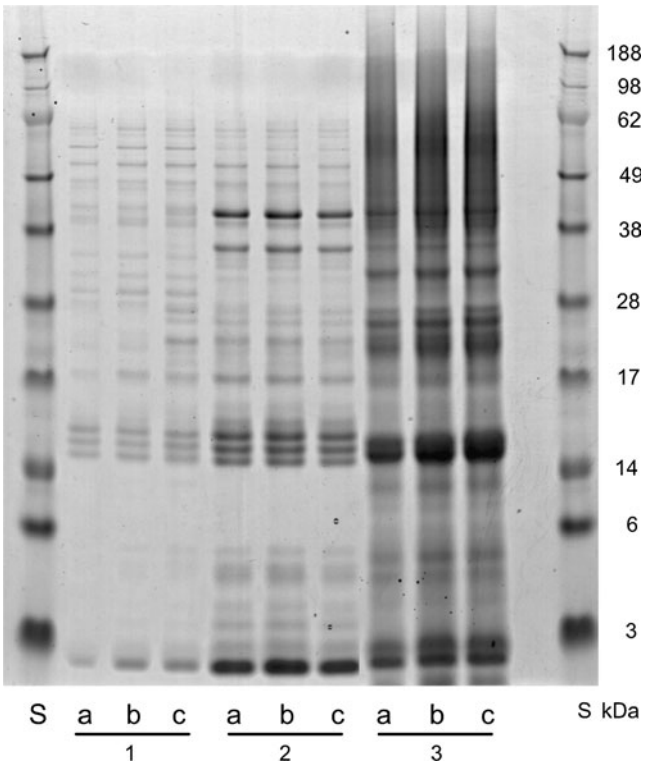

Fig. 2 SDS-PAGE analysis of Jatropha kernel proteins. Bands of proteins from Jatropha kernel showing molecular weight distribution after protein extraction from Jatropha kernel using (*1*) water, (*2*) 1.0 M NaCl, and (*3*) 0.055 M NaOH with different pretreatment: (**a**) non-heated, (**b**) preheated at 105 °C for 30 min, and (**c**) 121 °C for 30 min

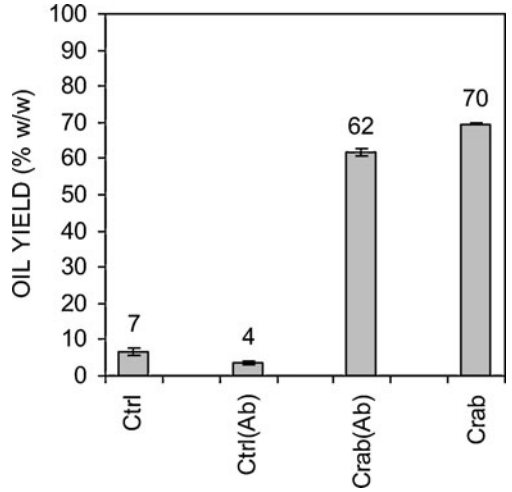

Fig. 3 Jatropha oil extraction yield from preheated kernel slurry incubated with paddy crab paste at 37 °C, 150 rpm for 24 h in comparison to control samples. Ctrl = control samples; Ab = antibiotics

sample resulted in even higher oil liberation (70%). The significant yield improvement from 62% to 70% indicates that mesophilic bacteria derived from paddy crabs take part in the entire mechanism of Jatropha oil liberation from pre-heated kernel. Based on these results, we decided to isolate mesophilic bacteria living in the gut of paddy crabs as our experimental strains for microbial Jatropha oil extraction.

Protease, xylanase, and glucanase activity of bacterial crude extract

We isolated 20 colonies of mesophilic crab bacteria, but we selected only seven colonies for further testing, namely strains MB4, MB5, MB7, MB11, MB12, MB13, and MB20 based on differences in colony form and microscopic observation. The detection of protease activity in crude extract revealed that MB4 is the only strain exhibiting protease, with different strengths of activity against the two types of protein tested: casein and *J. curcas* protein (Fig. 4a, b). The MB4 protease showed strong activity against casein as shown by a bright clear zone in the casein layer due to casein degradation by hydrolysis (Fig. 4a). However, the MB4 protease did not function with *J. curcas* protein under the conditions tested, as shown by the absence of a clear zone formed in the *J. curcas* protein layer (Fig. 4b). Xylanase activity was found only in MB4 crude extract as shown by formation of a clear zone in RBB-xylan agar medium (Fig. 4c). Congo Red staining in CMC agar medium showed a negative result for glucanase activity in the crude extract of all strains tested (Fig. 4d). Figure 4d depicts that the enzyme GC-220 (Genencor Inc, USA) lacked glucanase activity as no clear zone formed in CMC agar medium.

Selection of paddy crab bacterial strain for Jatropha oil extraction

Figure 5 shows the amount of oil extracted from 12 g preheated Jatropha kernel slurry (containing 2 g kernel), inoculated with the isolated bacterial strains directly prepared by suspending the NA culture slant with 2 ml water. We found that MB4 gave the highest Jatropha oil yield (63%), a 15-fold increase compared to a control experiment containing antibiotics. MB4 was selected for further tests at different conditions of incubations.

Identification of strain MB4

The phenotypical characterization conducted by DSMZ (Germany) indicates that MB4 is a *Bacillus pumilus* strain (Table 1). The partial sequencing of 16S rDNA (data not shown) conducted also by DSMZ shows a similarity of 100% to *Bacillus altitudinis* and 99% to the type strain of *B. pumilus*. The partial sequence has been submitted to the GenBank (accession number HQ860795). Considering the result of the partial sequencing, a clear identification to species level is not possible. Further examinations will be required to find out the novelty of the strain MB4. The

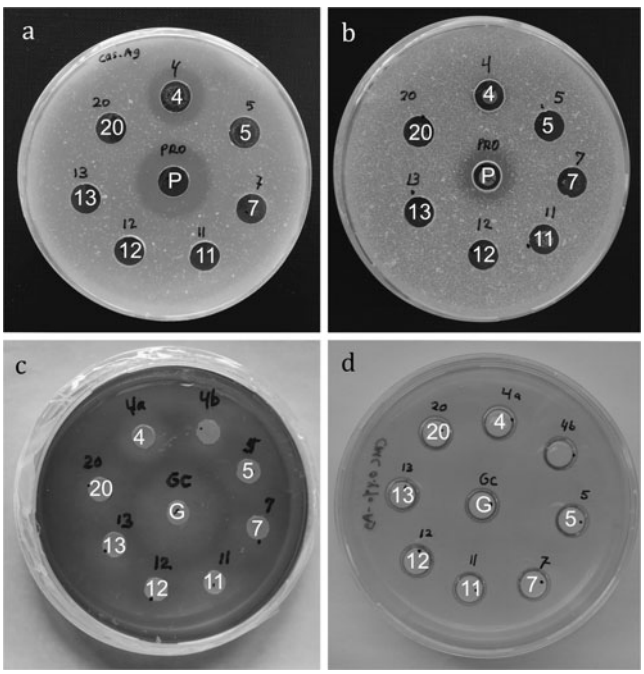

Fig. 4 Protease, xylanase, and glucanase activity of crude extracts of isolated bacterial strains on (**a**) casein agar plate, (**b**) Jatropha protein agar plate, (**c**) RBB-xylan agar plate, and (**d**) CMC agar plate. A 200x dilution of a protease, Protex 14 L (*P*) or a cellulase, GC-220 (*G*) from Genencor was the positive control. *Numbers* on the plates denote the strain: 4, 5, 7, 11, 12, 13, and 20 for MB4, MB5, MB7, MB11, MB12, MB13, and MB20, respectively

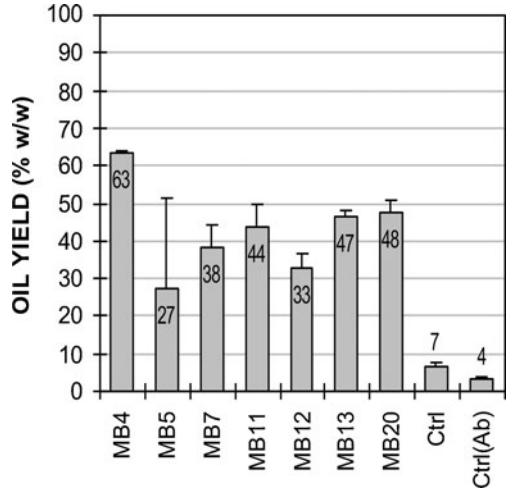

Fig. 5 Jatropha oil extraction yield from preheated kernel slurry after inoculation with different mesophilic bacterial strains from paddy crabs (MB4, MB5, MB7, MB11, MB12, MB13, and MB20) and incubated at 37 °C, 150 rpm for 24 h

strain MB4 has been deposited in the DSMZ collection as DSM 24473 *Bacillus* sp. BioMcc B-0081

Microbial Jatropha oil extraction: optimization

The optimization of microbially assisted oil extraction was conducted in three steps. In first instance, we incubated preheated Jatropha kernel slurry with the starter culture of strain MB4 at 37 °C over 24 h in order to find the optimum incubation time (Fig. 6a). Second, we studied the effect of initial pH (4.5, 5.5, 6.5, 7.5, and 8.5) of the kernel slurry on oil liberation by strain MB4; kernel slurry pH was adjusted to the desired value by using 4 M sodium hydroxide or 4 M sulphuric acid solutions before inoculation of bacteria starter cultures (Fig. 6b). Third, we optimized incubation temperature (37, 45, 50, and 55 °C) for Jatropha oil extraction (Fig. 6c).

Figure 6a shows that the oil yield in the control samples (containing antibiotics) remained below 10% throughout incubation for 24 h. The addition of MB4 starter culture to preheated kernel slurry resulted in a sharply increased oil yield to about 60% (tenfold increase compared to the control experiment) only within 6–8 h, after which it remained constant until 24 h. Based on these results, we decided to shorten the incubation time to 6 h in the subsequent experiments of microbial-assisted oil extraction.

The oil yield of kernel slurry incubated with MB4 for 6 h at different pH values (4.5, 5.5, 6.5, 7.5, and 8.5) is shown in Fig. 6b. The oil yield of MB4-treated sample increased from 65% at pH 4.5, to peak at 73% at pH 5.5, and then decreased to 50% at pH 8.5. Contrary to the curve trends obtained with MB4, the oil yield of control sample (containing antibiotics) decreased rapidly from 40% at pH 4.5 to 10% at pH 5.5, and then increased to 20% at pH 8.5. As a conclusion, strain MB4 has an optimum initial pH of 5.5 at 37 °C. Based on these results, we therefore studied the effect of incubation temperature on oil liberation by MB4 at pH 5.5 for 6 h.

The oil yield from kernel slurry incubated with strain MB4 for 6 h at pH 5.5 and different temperatures is shown

Table 1 Phenotypical characteristics of strain MB4

Observed item	Result
Shape of cells	Rods
Width (μm)	0.6–0.7
Length (μm)	2.0–3.0
Aminopeptidase test	−
KOH test	−
Catalase	+
Spores	Oval+
Sporangium swollen	−
Anaerobic growth	+
VP reaction	+
pH in VP	4.9
Growth temperature positive up to	50 °C
Growth in	
Medium pH 5.7	+
NaCl 2%	+
NaCl 7%	+
NaCl 10%	+
Lysozym 0.001%	+
Acid from	
D-Glucose	+
D-Fructose	+
D-Xylose	+
D-Mannitol	+
L-Arabinose	+
Gas from D-Glucose	−
Hydrolysis of	
Starch	−
Gelatin	+
Casein	+
Tween 80	+
Esculine	+
Lechitinase	+
Tyrosin degradation	−
Indol reaction	−
Use of	
Citrate	+
Propionate	−
Phenylalanine deaminase	−
Nitrate reduction	−
Arginine dihydrolase	−

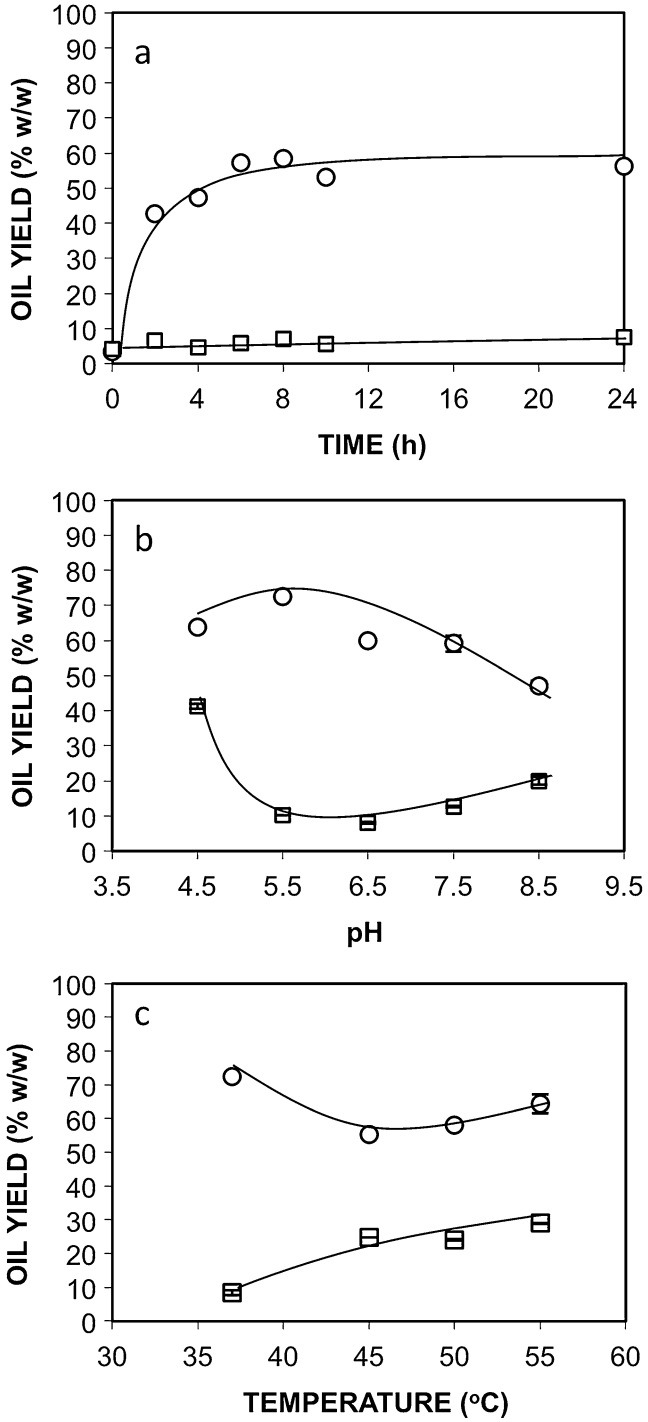

Fig. 6 Incubation of preheated Jatropha kernel slurry with MB4 (*white circle*) and without MB4 (*white square*): (**a**) oil extraction yield after incubation (37 °C for 24 h), the initial pH was not adjusted; (**b**) oil extraction yield after incubation at different initial pH (37 °C for 6 h); and (**c**) oil extraction yield after incubation at pH 5.5 at different temperatures (37, 45, 50, and 55 °C for 6 h)

in Fig. 6c. It is evident that the highest extraction yield of 73% was obtained at an incubation temperature of 37 °C. The oil yield of MB4-treated sample decreased from 73% to 60% as the temperature increased from 37 °C to 45 °C. The oil yield of MB4-treated sample slightly increased to 64% when the temperature increased from 45 °C to 55 °C. The oil yield of the control sample showed a slow increasing trend from 10% (37 °C), reaching a maximum oil yield of 30% only at 55 °C. This slow increase can at least partially explain the increasing trend of oil liberation in the MB4-treated sample at temperature in the 45–55 °C range.

Evaluation of protein integrity after microbial oil extraction

We investigated the molecular weight distribution of protein in liquid phase (supernatant) and solid phase (cake) after MB4 oil extraction, in comparison to those extracted with 0.055 M NaOH, by using SDS-PAGE analysis as shown in Fig. 7. We did not recover protein in the interfacial phase for SDS-PAGE analysis because we observed a very low amount of solid in the interfacial phase (between oil–water) after centrifugation, indicating a lower amount of oil–water emulsion after MB4 treatment.

Figure 7 shows that almost all proteins in the range of 1.0 to 88.5 kDa available in 0.055 M NaOH-extracted sample were also available in the solid phase, with the exception of one protein (88.5 kDa) that was missing in the solid phase. Three additonal proteins of 14.7, 27.4, and 44.9 kDa that were not available in NaOH-extracted sample were found in the solid phase as well as in the liquid phase. Six proteins of 1.7, 8.5, 9.4, 10.6, 11.3, and 32.1 kDa that

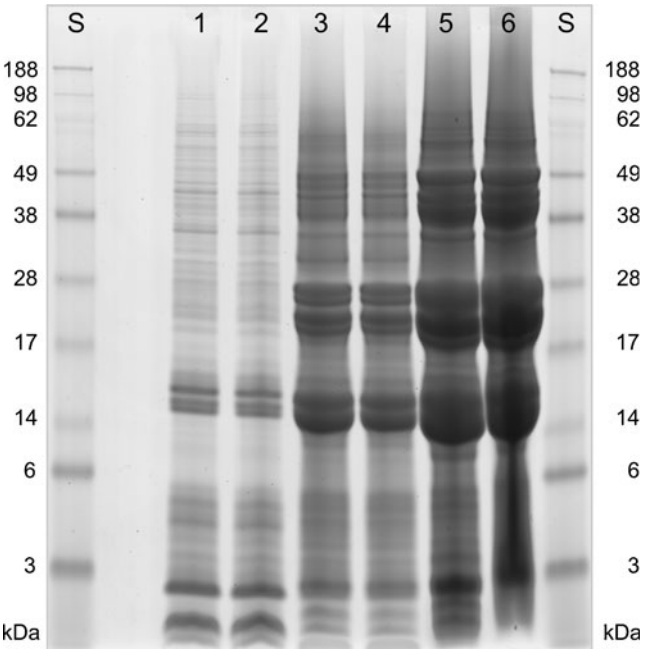

Fig. 7 SDS-PAGE analysis of MB4-treated Jatropha kernel proteins. Bands of proteins from duplicated samples of water phase (*1* and *2*), solid phase/cake (*3* and *4*) in comparison to Jatropha protein (*5* and *6*). *S* stands for standard of protein marker

were available in NaOH-extracted sample were not detected in the liquid phase. Furthermore, 13 additional proteins of 2.8, 11.9, 13.5, 14.7, 20.1, 25.1, 27.4, 29.2, 41.3, 44.9, 59.3, 100.1, and 130.4 that were not available in NaOH-extracted sample were found in the liquid phase.

The quality of oil after aqueous oil extraction

Oil quality data in Table 2 show that the oil obtained by MB4 extraction, in general, meet the German fuel standard DIN V 51605 for pure plant oil (rapeseed oil), except for the acid value (AV) which was found 8.6, or more than eight times higher than the standard value.

Discussion

J. curcas seed kernels have a high fat and protein content ranging between 45% and 55% *w/w* and 20–30% *w/w*, respectively (Gubitz et al. 1999; Lestari et al. 2010). The oil is investigated for its suitability as a biofuel, whereas the protein has been extensively studied for food and non-food application (Gubitz et al. 1999; Lestari et al. 2010; Lin et al. 2003; Martinez-Herrera et al. 2006). Therefore, with respect to the overall economy of Jatropha cultivation, it is important to find commercial outlets for both oil and protein.

In studying the effect of heat pretreatment on protein integrity, we found proteins resolution on electrophoresis gel gave identical band positions among non-heated, preheated at 105 °C for 30 min, and preheated at 121 °C for 30 min (Fig. 2). This means that the structure of Jatropha protein exhibits high thermal stability against thermal processing upon heating up to 121 °C for 30 min. Thermal properties of proteins are important to study the changes during heat processing which, in turn, are useful in the processing designs for protein-based products (Horax et al. 2011).

Aqueous extraction is necessary for the recovery of the protein from the kernel, and in order to decrease process costs it is therefore interesting to liberate the oil from the seed in the same step. In protease-assisted aqueous oil extraction from oilseeds, oil-bound proteins are hydrolyzed into smaller fractions, thereby altering their structure and functionality (Moure et al. 2006). Similar studies in Jatropha oil extraction reported previously did not highlight the importance of preserving protein structure during oil extraction process. If the protein structures are to be conserved to a large extent in the recovery of oil from oilseeds, the use of bacterial strains or enzymes liberating oil by other means than protein solubilisation is a reasonable choice.

Apart from proteases, a number of microbial enzymes have been studied to enhance oil extraction yields from oilseeds: amylase, glucanase, pectinase, cellulolytic, and hemicellulolytic enzymes (Dominguez et al. 1994). We were therefore interested to isolate and select other microbial strains from the crab's gut capable of assisting oil liberation without degrading protein.

The paste of paddy fields crabs are traditionally used for coconut oil extraction in Java. In a previous article, we have also applied paste crab to release oil from Jatropha kernels (Marasabessy et al. 2010). Whereas we now were able to release 70% of the oil, we previously only liberated 30% of the oil. Even though the experimental conditions in using paddy crab paste as the research material between the present study and the previous study (Marasabessy et al. 2010) look similar, they are not entirely the same for two reasons. First, in our present study, preheated kernels were used as substrate instead of non-heated kernel used in the previous study. Preheating the kernels might have enhanced the dissolution of cell components which were previously bound to the original structures of cells (Williams 2005), allowing crab's enzymes or microbial enzymes to have access in breaking oil barriers, resulting in the release of more oil as compared to control experiments (Fig. 3). Second, the different batch of crab paste used in the present study might have resulted in differences in oil liberation.

We found that MB4 starter culture was able to extract 73% oil from Jatropha kernel slurry when incubated for 6 h at 37 °C and pH 5.5. This is in good agreement with the Jatropha oil yield of 85.6% and 74% extracted by using

Table 2 The quality of oil extracted from Jatropha kernel using MB4 bacterial strain (AOE-MB4) compared to that extracted by expeller and the standard values

Parameters	Methods and oil quality		Standard value[a]
	AOE-MB4	Expeller	
Seeds source	*J. curcas*	*J. curcas*	–
Feed type	Preheated kernels	Whole seeds	–
Expeller type	–	De-Smet UK	–
Conditions	6 h, 150 rpm, 37 °C	25 rpm, 80–85 °C	–
OSI (h)	7.8±0.06	10.7	Min 6
AV (mg KOH/g oil)	8.6±0.20	10.3	Max 2
Water (mppm)	719±32	1147	Max 750

OSI oxidative stability index, *AV* acid value

[a] German Fuel Standard DIN V 51605

protease of Alcalase (Novo Nordisk, Denmark) and Protizyme (Jaysons Agritech, India), respectively (Shah et al. 2005; Winkler et al. 1997). The use of Viscozyme (Novo Nordisk, Denmark) as a hemicellulase/cellulase formula gave a comparable oil yield of 70% (Winkler et al. 1997).

We have shown that protease from strain MB4 bears no activity against Jatropha protein. Hence, by considering the optimal pH and temperature of MB4 (pH 5.5 and 37 °C, respectively) and also the presence of xylanase in the crude extract of MB4, it is most likely that the strain MB4 facilitates oil liberation at 37 °C via the degradation of hemicellulose that forms the oil-containing cell wall structure of the kernel (Rosenthal et al. 1996).

Bacterial identification results suggested the strain MB4 as *B. pumilus* or the closely related *B. altitudinis*. In case of *B. pumilus*, previous investigations have reported the potential application of *B. pumilus* as xylanase producer (Ahlawat et al. 2007; Battan et al. 2007; Kapoor and Kuhad 2007; Kapoor et al. 2008; Nagar et al. 2010; Wang et al. 2010; Yasinok et al. 2010). In contrast, we found that only a few publications are available on the potential application of *B. altitudinis*.

After MB4-assisted oil extraction, the extracted oil has an AV below 14% (Table 2), which seems applicable for biodiesel production since a chemical pretreatment to reduce the acid value from 14% to 1% before transesterification of Jatropha oil into biodiesel has been established recently, which results 99% yield of biodiesel (Tiwari et al. 2007).

Concluding, strain MB4 identified as *B. pumilus* or *B. altitudinis* isolated from paddy crab liberated 73% *w*/*w* of Jatropha oil from preheated kernel in aqueous system after 6 h incubation at 37 °C. It is suggested that the strain MB4 facilitates oil liberation via degradation of hemicellulose. Incubation of *J. curcas* kernel with strain MB4 preserves the Jatropha protein structure to a large extent. MB4-assisted oil extraction has several advantages: (a) no purified cocktail enzyme preparation is required, (b) protein integrity is mostly preserved, and (c) this method results in Jatropha oil with a quality which is suitable for biodiesel production.

Acknowledgments The authors gratefully acknowledge the Koninklijke Nederlandse Akademie van Wetenschappen, Scientific Programme Indonesia—Netherlands (SPIN-KNAW) for the financial assistance. We also would like to acknowledge Erna Subroto, PhD student at Rijks Universiteit Groningen the Netherlands for conducting oil quality analysis, and Dianika Lestari for her help in conducting gel electrophoresis of Jatropha protein of our samples.

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